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Antibiotic-induced socio-sexual behavioral deficits are reversed via cecal microbiota transplantation but not androgen treatment

Stephanie Salia, Yellow Martin, Francine F. Burke, Leah A. Myles, Lauren Jackman, Katherine Halievski, Francis R. Bambico, Ashlyn Swift-Gallant *

Department of Psychology, Memorial University of Newfoundland, 232 Elizabeth Ave. St. John's, NL, A1B 3X9, Canada

A B S T R A C T

Recent evidence has demonstrated a sex-specific role of the gut microbiome on social behavior such as anxiety, possibly driven by a reciprocal relationship between the gut microbiome and gonadal hormones. For instance, gonadal hormones drive sex differences in gut microbiota composition, and certain gut bacteria can produce androgens from glucocorticoids. We thus asked whether the gut microbiome can influence androgen-dependent socio-sexual behaviors. We first treated C57BL/6 mice with broad-spectrum antibiotics (ABX) in drinking water to deplete the gut microbiota either transiently during early development (embryonic day 16-postnatal day [PND] 21) or in adulthood (PND 60–85). We hypothesized that if ABX interferes with androgens, then early ABX would interfere with critical periods for sexual differentiation of brain and thus lead to long-term decreases in males' socio-sexual behavior, while adult ABX would interfere with androgens' activational effects on behavior. We found that in males but not females, early and adult ABX treatment decreased territorial aggression, and adult ABX also decreased sexual odor preference. We then assessed whether testosterone and/or cecal microbiota transplantation (CMT) via oral gavage could prevent ABX-induced socio-sexual behavioral deficits in adult ABX-treated males. Mice were treated with same- or other-sex control cecum contents or with testosterone for two weeks. While testosterone was not effective in rescuing any behavior, we found that male CMT restored both olfactory preference and aggression in adult ABX male mice, while female CMT restored olfactory preference but not aggression. These results suggest sex-specific effects of the gut microbiome on socio-sexual behaviors, independent of androgens.

1. Introduction

Socio-sexual behaviors are essential for rodent reproductive success (Huo et al., 2018; Swaney et al., 2012). These behaviors and their neural substrates exhibit robust sex differences that are regulated by input from sensory systems such as the accessory olfactory system as well as by reward circuits and hormone signaling (Chu et al., 2015; Gunaydin et al., 2014). In particular, gonadal hormones (e.g., testosterone) act via steroid receptors in the brain during early critical periods of development and in adulthood to drive the development and presentation of socio-sexual behaviors including male-typical sexual behavior (e.g., mounting) and territorial aggression (Phoenix et al., 1959; reviewed in Arnold, 2009). Disruption of androgen exposure in male mice, for instance, can result in a lasting decrease in male-typical behaviors (Bodo and Rissman, 2008; Monks and Swift-Gallant, 2018), whereas increasing androgens via testosterone treatment can augment male-typical behaviors in females (Martel and Baum, 2009). Indeed, the role of androgens in regulating sex-typical socio-sexual behaviors is well established. However, emerging evidence suggests that sex-dependent behaviors can also be influenced by other peripheral factors—namely, the gut microbiome—via gut-brain interactions (Mayer et al., 2014; Sylvia et al., 2017).

In previous research, the gut microbiome, which includes protozoa, archaea, eukaryotes, viruses, and bacteria living in the gastrointestinal tract, has been shown to play an important role in health and disease (Cryan and Dinan, 2012; Jašarević et al., 2016). Specifically, the gut microbiota communicates with the brain via the gut-brain axis by integrating neuroendocrine and metabolic pathways, affecting the brain and behavior (Carabotti et al., 2015; Clarke et al., 2013; Gwak and Chang, 2021; Thursby and Juge, 2017; Zhu et al., 2017). For instance, alterations in the microbiota composition (i.e., dysbiosis) induced by either pathogenic infections or antibiotic (ABX) treatment have been shown to increase anxiety-like behavior, decrease social interaction and exaggerate stress responsivity in mice (Bercik et al., 2011; Buffington et al., 2016; Desbonnet et al., 2014; Gacias et al., 2016; Diaz Heijtz et al., 2011; Jašarević et al., 2016; Sylvia et al., 2017; Wu et al., 2021). It has also been shown that the microbiome mediates behaviors in a sex-dependent manner, suggesting that sex hormones may play a role in this process (Jašarević et al., 2016; MacFabe et al., 2011; Sylvia et al., 2017). In mice, sex differences in the gut microbiota composition appear

* Corresponding author.
E-mail address: aswiftgallant@mun.ca (A. Swift-Gallant).

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at puberty, where males show a lower bacterial diversity and richness than females (Elderman et al., 2018; Menon et al., 2013; reviewed in Kim et al., 2020), that is dependent on androgens. Eliminating the primary source of androgens in male mice through castration shifts males' microbiota closer to that of females than noncastrated males (Yurkovetskiy et al., 2013). These studies suggest gonadal hormones shape the microbiota composition, which may translate into sex-dependent microbial-mediated behavioral deficits. In turn, the gut microbiota can also alter sex hormonal levels (Colldén et al., 2019; Markle et al., 2013), such as through the gut microbe *Clostridium scindens,* which can convert glucocorticoids into androgens (Ridlon et al., 2013). Moreover, it has been shown that ABX treatment, which significantly alters the gut microbiota, also decreases testosterone levels in male rats (Popoola et al., 2014). This reciprocal relationship between the microbiota and sex hormones collectively suggests that the microbiota can influence the production and metabolism of androgens——the primary class of sex hormones that mediate sexual differentiation of the brain and socio-sexual behaviors in mice (Arnold (2009).

Recent work in Siberian hamsters has shown that the gut microbiome affects social behavior in a sex-dependent manner (Sylvia et al., 2017). In their study, a single ABX treatment was sufficient to reduce aggression in female hamsters, whereas two treatments were necessary to do so in male hamsters, indicating that the gut microbiome mediates aggression differently in male and female hamsters. Given the role of androgens on the display of male-typical aggression in mice (e.g., castration of a male decreases aggression) and the associations between sex hormones and the microbiota, we hypothesized that the microbiome may influence socio-sexual behaviors (e.g., sexual odor preference and territorial aggression) in a sex-dependent manner via androgen-dependent pathways in rodents. Thus, we aimed to characterize the extent of microbiota mediation on sex-typical socio-sexual behaviors to further elucidate the range of factors that influence sex differences in the brain and behavior.

In the present study, we asked whether depletion of the gut microbiota via broad-spectrum ABX alters socio-sexual behaviors in mice and whether the gut microbiome acts through androgens to mediate these behaviors. We hypothesized that depletion of the gut microbiota (via ABX) in early development and/or adulthood would have sex-dependent effects on sex-typical behaviors in mice. Specifically, it was hypothesized that gut microbiome depletion in early development may have long-lasting effects when administered during early critical periods for sexual differentiation of brain and behavior (i.e., during the male-typical perinatal androgen surge), whereas adulthood microbiota depletion may impact the activational/transient effects of androgens for sex-typical behaviors. We also predicted that recolonizing the gut microbiota and/or treating mice with testosterone would reverse ABX-induced behavior changes in adult male mice. Our results support a transient sex-dependent role of the gut microbiome for sex-typical behaviors in male mice; however, to our surprise, these effects appear to be independent of androgens.

2. Materials and methods

2.1. Animals

Animal use and experiments were conducted following the Canadian Council on Animal Care (CCAC) guidelines and approved by the Institutional Animal Care Committee at Memorial University of Newfoundland and Labrador. Male and female mice were generated in-house by breeding C57BL/6 mice (Charles River Laboratories, QC, Canada). Offspring were group-housed (2–3 mice per cage) at weaning (postnatal day [PND] 21–22) and individually housed from PND 50. Mice had *ad libitum* access to a standard chow diet (Teklad Global Rodent Diets Envigo) and water and were kept on a 12-h light-dark cycle with lights on at 7 a.m.

Male and female mice from the same litter (4–6 litter per group) were randomly assigned to one of the following treatment groups to determine the effect of the microbiota depletion on socio-sexual behaviors (see Fig. 1a for experiment timeline): Vehicle control group (n = 11 males, n = 8 females) in which animals received purified water without ABX, Adult ABX group (n = 14 males, n = 11 females) in which animals received ABX in adulthood from PND 60–85 (for at least 20 days before behavior testing, as well as throughout behavior testing), or Early ABX group (n = 18 males, n = 13 females) where animals received ABX via maternal exposure from embryonic day 16-PND 21. Embryonic day 16 was determined by daily examination of breeder females for a seminal plug to approximate the first gestation day.

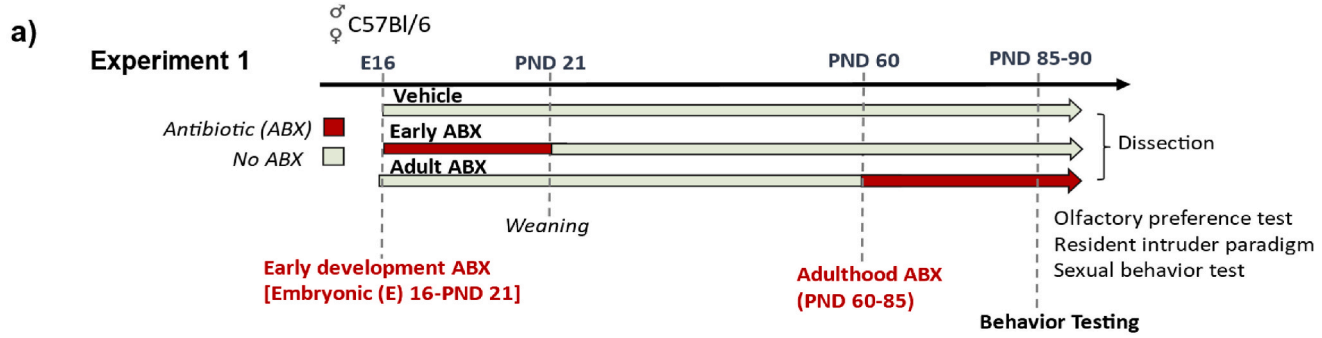
Next, we assessed the potential pathway by which the microbiota modulate male-typical socio-sexual behaviors in mice. Based on our earlier finding that ABX males, but not females mice exhibit socio-sexual behavioral deficits, with larger deficits in adult ABX males, only male mice were used in the follow-up study (Fig. 1c). Adult male mice were assigned to one of the following conditions at random: 1) Vehicle-treated control males (no ABX treatment, saline gavage/blank capsule implant, n = 12); 2) ABX (males that received ABX only, saline gavage/blank capsule implant, n = 12); 3) MC (ABX-treated males that received a cecal transplant from healthy/untreated male controls/blank capsule implant, n = 12); 4) FC (ABX-treated males that received a cecal transplant from healthy/untreated female controls/blank capsule implant, n = 9); and 5) ABX + T (testosterone capsule implant in ABX males, n = 12). T capsules were implanted on PND 55, while ABX treatment started on PND 60. Mice in the MC and FC groups received two weeks of ABX prior to microbiota transplantation (i.e., PND 75–86), while the ABX and ABX + T conditions received ABX until the end of the experiment (i.e., PND 60–90).

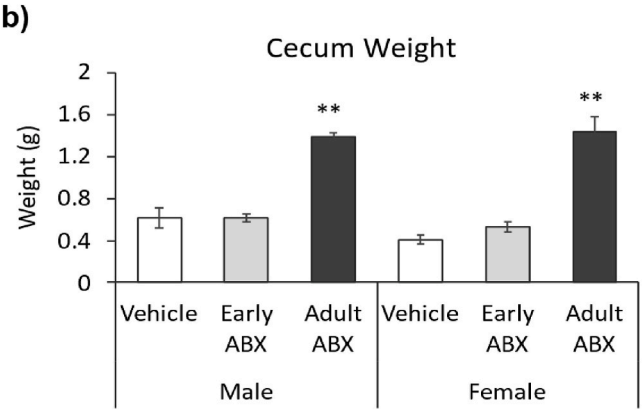
Stimulus animals

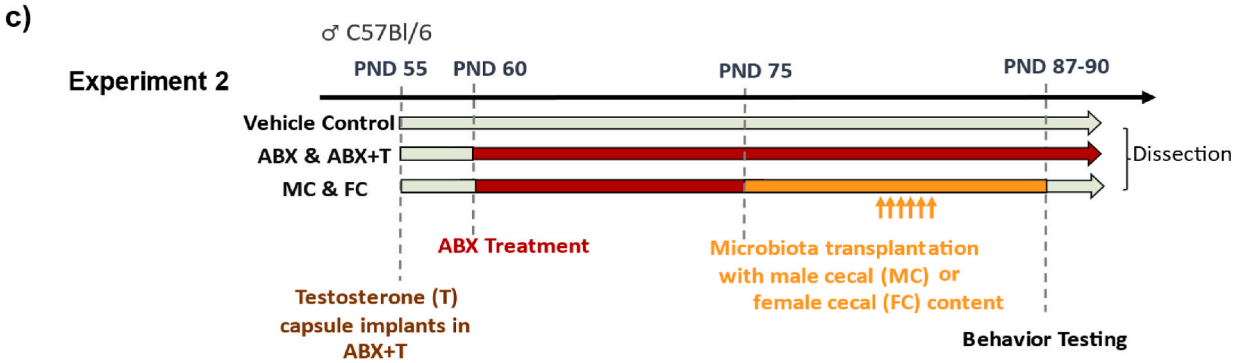
Male and female C57Bl/6 gonadectomized mice were utilized as stimuli during behavior testing. At 9–12 weeks of age, male stimulus mice were castrated, and females were ovariectomized and subcutaneously implanted with a Silastic capsule (1.98-mm inner diameter/3.17-mm outer diameter; 6-mm effective release length) containing dissolved 17β-estradiol in sesame oil (50 μg in 0.025 ml), which was sealed with Silastic Medical Adhesive Silicone (Dow Corning, Midland, MI, USA). Stimulus mice were housed in groups of 2–4 per cage. To induce behavioral estrus, stimulus females were injected with progesterone (500 μg in 0.1 ml of corn oil) 2–5 h before sexual behavior testing (Swift-Gallant et al., 2016a). Urine was pooled from 5 to 8 male mice (aliquots were frozen at −20 °C and thawed on testing days before each resident-intruder test). Gonadectomized stimulus males were swabbed on their lower back/near their tail with 40–50 μl urine collected from sexually experienced males–such urine has been shown to contain pheromones that elicit inter-male aggression (Mucignat-Caretta et al., 2004). Soiled bedding used in the olfactory preference test was collected from sexually experienced males and estrus-induced females 48 h following cage change. The bedding was stored in resealable bags at −20 °C and was allowed to reach room temperature before use on the testing day.

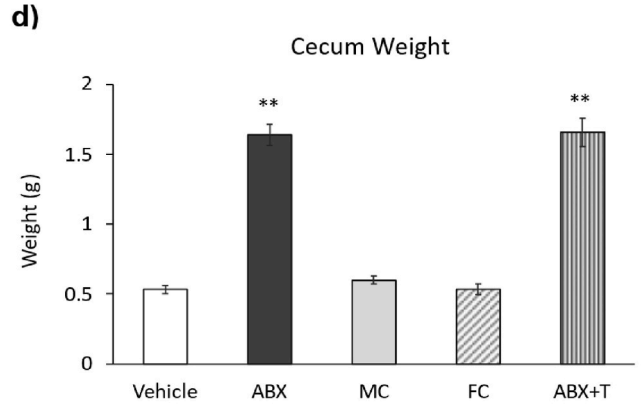
2.2. ABX treatment

Mice received broad-spectrum ABX in drinking water (1 g/L ampicillin, 1 g/L neomycin sulphate, 500 mg/L vancomycin, and 10 mg/L erythromycin from Cayman Chemical Company, and 100 mg/L gentamicin from Tocris Bioscience) for 2–4 weeks to deplete the microbiota (Sampson et al., 2016). When administered for 2–4 weeks, this combination of ABX has been reported as safe and sufficient to deplete the microbiota (Reikvam et al., 2011; Sampson et al., 2016). Notably, mice receiving cecal transfer (i.e., MC and FC groups) were given ABX in drinking water for 14 days, which has been shown to induce a robust and consistent depletion of the gut microbiota in as little as 4 days of treatment (Tirelle et al., 2020; Sylvia et al., 2017). ABX treatment was subsequently discontinued in MC and FC mice prior to cecal transfer as









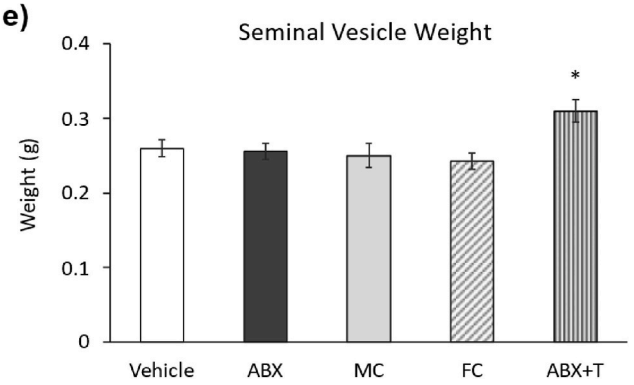


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Fig. 1. Microbiota transplantation reversed antibiotic-mediated microbiota depletion. (**a**) Timeline schematic for the effect of antibiotic (ABX) treatment in early development and adulthood on the microbiota and behavior: Early ABX mice ($n = 18$ males, $n = 13$ females) received ABX from embryonic day 16-PND 21) via maternal exposure/drinking water while Adult ABX ($n = 14$ males, $n = 11$ females) received ABX from PND 60 to end of the experiment via drinking water (i.e., PND 85). (**b**) Adulthood ABX led to a significantly enlarged cecum in male and female mice, indicative of microbiota depletion. (**c**) Timeline schematic for the effects of male or female control microbiota transplant or testosterone (T) treatment in ABX-treated male mice: T capsules were implanted in the ABX + T mice on PND 55. ABX ($n = 12$) and ABX + T ($n = 12$) male mice received ABX from PND 60 until the end of the experiment. MC ($n = 12$) and FC ($n = 9$) male mice received two weeks of ABX treatment followed by six treatments of CMT with either same (MC) or opposite-sex (FC) microbiota every other day via oral gavage. (**d**) ABX treatment for 3–4 weeks significantly depleted the microbiota in ABX and ABX + T male mice. MC and FC male mice were comparable to vehicle-treated control males. (**e**) T treatment increased androgen-sensitive seminal vesicle weight in ABX + T compared to MC and FC males but not vehicle-treated control. Data represent mean ± SEM; $^{**}p < .001$ and $^{*}p < .05$ indicate a significant main effect of ABX treatment and significant post hoc test when compared to vehicle controls.

that would negate the effects of cecal transfer.

2.3. Testosterone (T) treatment

Silastic capsules containing T (1.6-mm inner diameter, 3.2-mm outer diameter, 6-mm effective release length) were subcutaneously implanted in the ABX + T group. In castrated male mice, capsules of this length result in a near-normal level of circulation T (Chen et al., 2014; Zuloaga et al., 2008). All other groups (Vehicle, ABX, MC and FC) had blank capsule implants to control for surgical implant stress.

2.4. Cecal microbiota transplantation (CMT)

Cecal contents used for microbiota transplantation were harvested from the ceca of 8-9-week-old healthy/untreated control male and female mice (n = 6–8/group) into sterile 2-ml microcentrifuge tubes. Same-sex cecal contents were mixed to ensure homogeneity, diluted with 1 × phosphate-buffered saline (PBS) in a 1:1 ratio and stored at −80 °C until the day of transplantation (i.e., less than 2 months between cecal microbiota collection and transplantation; Le Bastard et al., 2018; Ubeda et al., 2013). Short-term storage for this duration has been shown to preserve anaerobic biomass with or without cryoprotection (Rothrock et al., 2011). Additionally, we stored cecal microbiota in aliquots to eliminate the need for repeated freeze-thaw cycles, which is shown to contribute to DNA extraction degradation (Poulsen et al., 2021). On the day of the CMT, aliquots were thawed and further diluted with 1 × PBS at a 1:5 ratio for a final dilution of 1:10. For recolonization of the gut following the 2-week ABX treatment period, MC and FC male mice received 200 μl of male cecal and female cecal content, respectively, via oral gavage every other day for 12 days (for a total of 6 treatments). Vehicle control, ABX and ABX + T male mice also received 200 μl of 1 × PBS to control stress induced by oral gavage.

2.5. Behavioral testing

A battery of tests assessing sexual and aggressive behaviors was conducted on each mouse, during the light phase between 9 a.m. and 4 p.m. on three consecutive days, with the olfactory preference test occurring on the first day. Resident-intruder and sexual behavior tests were counterbalanced between days 2 and 3. All experimental mice were sexually naïve at the time of testing.

2.5.1. Olfactory preference test

A bedding preference test was used to assess opposite- and same-sex odor preferences. The test was performed as follows: experimental animals were placed in a clear container (42 × 25 × 20 cm) with clean bedding covering the bottom and three ramekins (4-cm height and 8-cm diameter) containing clean bedding for a 5-min habituation phase. Immediately after habituation, the bedding in the ramekins were replaced with male-soiled, female-soiled, or clean bedding in a quasi-randomized order. Testing phase trials were video-recorded for 10 min after the experimenter left the testing room. Video recordings were analyzed by a blinded experimenter for the duration of time each mouse spent investigating each ramekin using the Behavioral Observation Research Interaction Software (BORIS©, Turin, Italy). Ramekins were later matched with the corresponding bedding type using the testing records, and a female preference score (i.e., time spent in female bedding minus time spent in male bedding) was calculated.

2.5.2. Resident-intruder paradigm and sexual behavior test

All mice were tested on the resident-intruder paradigm, which measured territorial aggressive and social interaction behaviors towards a urine-swabbed gonadectomized male intruder. Experimental mice underwent a second test to measure sexual behaviors in response to an estrus-induced female intruder. Both tests were conducted in the experimental animal's home cage by taking out all enrichment and food, leaving only the bedding, and the cage lid was replaced with a transparent Plexiglass lid with air holes. Behaviors were video-recorded for 15-min once experimenters left the room. The frequencies and durations of socio-sexual behaviors, including anogenital investigation, face/body investigation, self-grooming, mounting, thrusting, and intromission were measured. Aggression was defined as the number and duration of chasing, pounce/attack, tumbling, boxing, and biting. Latencies to the first sexual and aggressive behavior were recorded with a maximum score of 900 s given to mice that did not show any sexual or aggressive behavior. An experimenter blind to the sex of the experimental and stimulus mice analyzed videos for behaviors using BORIS©.

2.6. Tissue harvesting

At the end of each experiment, mice were transcranially perfused using 0.1 M phosphate buffer followed by 4% paraformaldehyde in 0.1 M PBS. Body weight and the weights of seminal vesicles and testes (males) and uterine horn and ovaries (females) were measured. Whole ceca were also collected and weighed to validate the microbiota depletion via ABX (i.e., an enlarged cecum depicts microbiota depletion; Reikvam et al., 2011). Brains were extracted and post-fixed for 2 h in 4% paraformaldehyde and then transferred to 20% sucrose, where they were stored at 4 °C until sectioning. Cecal contents were collected during dissection and stored at −80 °C for bacterial sequence analysis.

2.7. 16S bacterial gene sequencing and analysis

Bacterial sequencing was performed on cecal contents collected from treated test mice (ABX, MC, FC and ABX + T) and untreated control (i.e., no ABX or CMT) male and female mice to enable a sex-based comparison of bacterial abundance. As both the female and male control groups were from the same colony (inbred mice with similar housing and feeding conditions), it was expected that their gut microbiota would be similar to that of the donor mice (Hufeldt et al., 2010). Frozen fecal samples were shipped on dry ice to Integrated Microbiome Resource (Dalhousie University, Halifax) for DNA sequencing and bioinformatics. Bacterial gene amplicons were generated using the new Microbiome Helper pipeline based on the open-source pipeline Quantitative Insights into Microbial Ecology version 2 (QIIME 2.0) (Bolyen et al., 2019) and subsequently normalized for analysis. Sequencing of samples yielded a total of 392,018 reads (median = 6602; maximum = 85,365; minimum = 1279), with an average of 17,044, reads per sample. Notably, sequencing could not be profiled on fecal samples with low biomass following ABX depletion; hence group sample size decreased for the

bacterial composition analysis: Female control, $n = 6$; Male control, $n = 8$; MC, $n = 4$; FC, $n = 4$; ABX + T, $n = 1$; and ABX, $n = 0$. Shannon alpha diversity was performed using Kruskal-Wallis to determine microbiota diversity, and beta diversity Principal coordinate analysis (PCoA) plots were generated based on Weighted UniFrac estimation, as implemented in QIIME2. Because the group sample size was too small for continued analysis following low sequencing reads (i.e., < 1000 reads), ABX ($n = 0$) and ABX + T ($n = 1$) groups were excluded from subsequent analyses on taxonomic differences at the phylum and genus levels and were assessed by an Analysis of Variance (ANOVA) in the statistical software Jamovi (version 1.6.23; Open-source Project., Sydney, Australia).

2.8. Statistical analysis

We analyzed all somatic and behavioral variables using ANOVA in Jamovi and attributed statistical significance at $p < .05$. All significant omnibus effects were followed with Tukey post-hoc analyses.

3. Results

3.1. CMT with male or female microbiota reverses gut dysbiosis in adult ABX-treated male mice

Our first goal was to determine whether depletion of the gut microbiota in mice via ABX treatment during early development (embryonic day 16-PND 21) or in adulthood (PND 60–85) mediated sex-typical behavior in mice. To validate the depletion of the microbiota, we weighed the ceca of ABX-treated and untreated vehicle-control mice and found a significant effect of ABX treatment on cecum weight ($F(2, 67) = 70.90$, $p < .001$, $\eta p^2 = 0.679$; Fig. 1b). Specifically, Adult ABX but not Early ABX male and female mice had significantly heavier ceca compared to vehicle control mice ($p < .001$), suggesting that ABX effectively depleted the microbiota in adulthood. This is consistent with a previous report that enlarged and heavier ceca are a macroscopic sign associated with gut microbiota depletion (Reikvam et al., 2011). On the contrary, the ceca of Early ABX mice were comparable in size to vehicle controls ($p > 0.05$), likely because the Early ABX mice had nearly 64+ days to recover post-ABX treatment. Nevertheless, ABX exposure at a critical development window may have long-term consequences on the brain and behavior. For instance, maternal ABX treatment during gestation has shown profound alterations in the composition of microbiota and immunity in offspring, translating into behavioral deficits both in early life and adulthood (Nyangahu et al., 2018; O'Connor et al., 2021). Our next goal was to evaluate whether CMT with same- (MC) or opposite-sex (FC) control mice or testosterone treatment (ABX + T) could reverse the ABX-induced gut changes in adult male mice. Here, we found a significant treatment effect on cecum weight ($F(4, 52) = 87.6$, $p < .001$, $\eta p^2 = 0.871$; Fig. 1d). In line with our initial result, ABX and ABX + T male mice that were given 3–4 weeks of ABX treatment had heavier ceca than vehicle control, MC, and FC (all p values < .001). In contrast, ABX-treated males that received microbiota transplant from either male or female control mice (MC and FC, respectively) had a lower cecum weight comparable to untreated vehicle controls (all p values >.500), suggesting a successful recolonization of the depleted microbiota via CMT.

To determine the breadth of the microbial recolonization with CMT, we compared 16S rRNA bacterial sequence results from test mice that were reconstituted with male control (MC) or female control (FC) microbiota via oral gavage to untreated (no ABX or CMT) female and male control mice. Shannon alpha diversity rarefaction index showed no significant differences in bacterial diversity ($H(4) = 2.84$, $p = .586$; Fig. 2a), indicating reconstitution of relative bacterial abundance in MC and FC groups. Our beta diversity measure of similarity was based on weighted UniFrac, which takes into account the relative abundance of taxa shared between samples (Lozupone et al., 2011). When viewed on the PCoA plot, all groups ordinated closer to one another, although the female control mice clustered at the mid-upper right side of the plot (above male control and CMT male mice), whereas untreated control males grouped at the bottom-right, suggesting a potential sex difference in bacterial similarity (Fig. 2b). On the other hand, MC and FC male mice were similar in that they clustered together (far right bottom) regardless of CMT type received and were closer to male controls than female controls suggesting that host-specific factors drive the reshaping of the bacterial communities following CMT. Overall, the microbiota composition of all samples (control females, control male, MC, and FC) was dominated by the phyla Bacteriodetes (74.40%), followed by Firmicutes (25.31%) and showed no significant differences between CMT groups and control mice (all p values >.100; Fig. 2c). Also, we determined the relative bacterial abundance at the genus level (Fig. 2d), which revealed a significant difference in *Lactobacillus* abundance ($F(3, 18) = 4.33$, $p < .018$, $\eta p^2 = 0.419$). The female control group had significantly more *Lactobacillus* genera than either males receiving MC or FC microbiota (all p values <.05; Fig. 2e). Male controls showed no significant difference from female controls but did show a trend towards reduction ($p = .083$), suggesting sex differences in *Lactobacillus* abundance. The genera *Clostridium scindens,* which have been linked with androgen metabolism (Ridlon et al., 2013), was not detected in all samples examined. Together, the analysis of the microbiota composition after ABX and CMT treatments confirmed that ABX-induced microbiota depletion was successful in our study and can be restored by 6 treatments of CMT over a 2-week period regardless of the sex of the donor.

3.2. ABX treatment decreased male-typical olfactory preferences but not sexual behaviors in mice

Once we confirmed that ABX treatment successfully depleted the microbiota in mice, we examined whether this intervention affected sexual odor preference by conducting the olfactory preference test. We calculated the female preference score (i.e., time spent in female-soiled bedding minus time spent in male-soiled bedding) and found that ABX treatment significantly decreased the female preference score ($F(2, 69) = 5.44$, $p = .006$, $\eta p^2 = 0.136$; Fig. 3b). Specifically, Adult ABX mice had a significantly lower female preference score than those in the Early ABX group ($p = .046$) and vehicle control mice ($p = .007$), but Early ABX mice were comparable to vehicle controls ($p > .100$). We also observed a main effect of sex on female preference score, where male mice displayed a greater preference for female-soiled bedding than female mice ($F(1, 69) = 38.43$, $p < .001$, $\eta p^2 = 0.358$). Given the observed main effect of sex and our *a priori* predictions regarding sex and the gut microbiome, we conducted a sex-based analysis on time spent investigating each soiled-bedding type (i.e., male-soiled, female-soiled, and neutral bedding) to assess whether males or females in the Adult ABX group were driving the main effect of ABX on the female preference score. We did not find any significant differences between Adult ABX females and vehicle control females in the time spent investigating each soiled-bedding type (all p values > .100; Fig. 3c). However, Adult ABX males spent significantly more time investigating male-soiled bedding than vehicle control males ($p = .007$), but there were no differences in time spent investigating female-soiled or neutral bedding. These results suggest that ABX in adulthood affected olfactory preference in male mice, driven by an increase in the investigation of male-soiled bedding rather than a decrease in the investigation of female-soiled bedding.

We also asked whether the increased investigation of male-soiled bedding among Adult ABX males would translate into decreased investigative and sexual behaviors when presented with a live stimulus female in the sexual behavior test. We found that this was not the case and that ABX did not affect investigative and sexual behaviors towards an estrus female intruder (all p values > .100), though the expected sex effect was found where male mice mounted, thrusted, and intromitted estrus-induced female intruders to a greater extent than female mice, regardless of ABX condition (all p values < .001). In response to a urine-swabbed castrated male intruder, we found a main effect of ABX on the

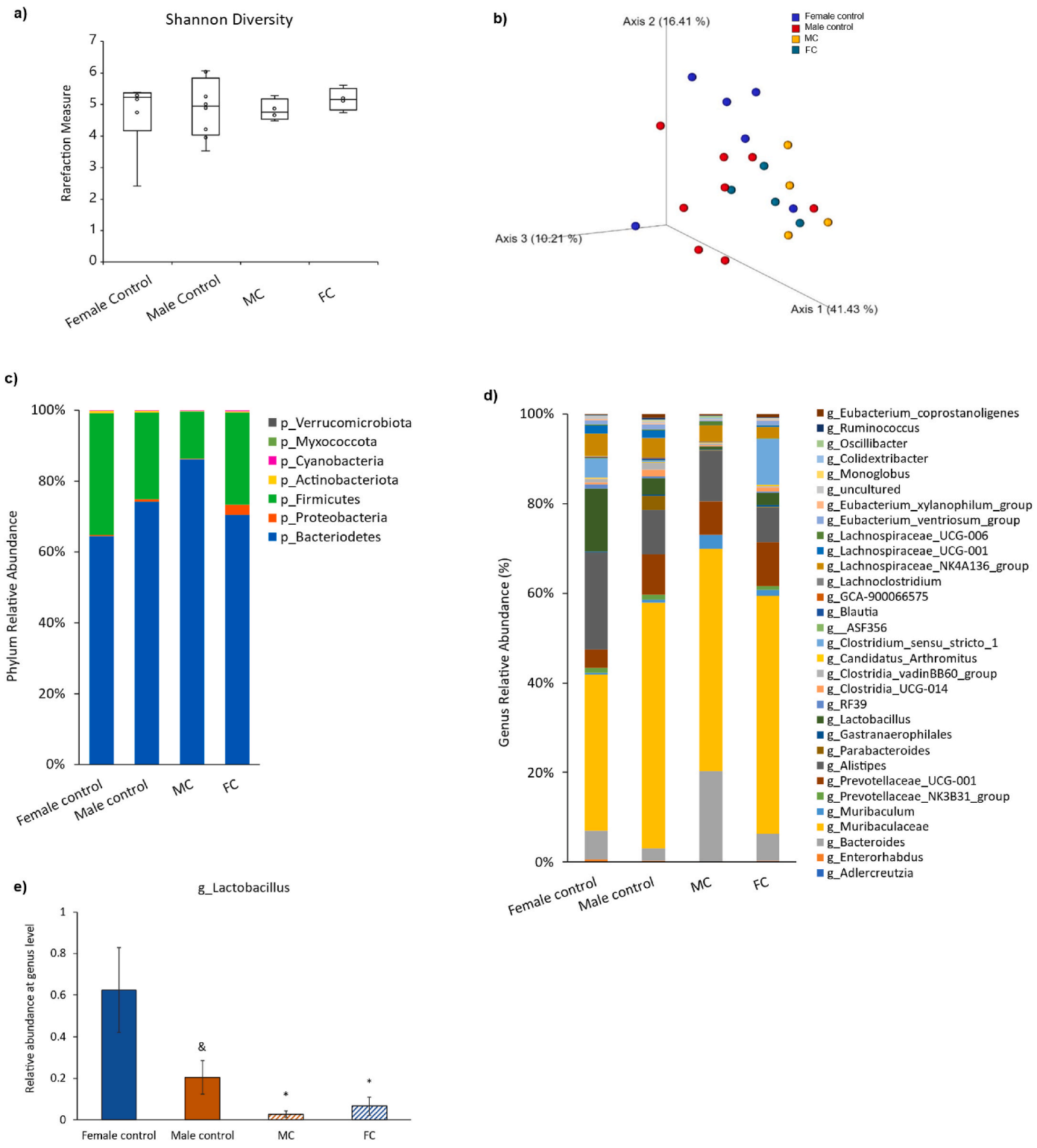

Fig. 2. Bacterial alpha and beta diversity plots and relative bacterial abundance at the phylum and genus levels. **(a)** 16S rRNA sequencing analysis of fecal samples from untreated female control, untreated male control, MC and FC male mice. **(a)** Boxplot showing Shannon alpha diversity rarefaction index calculated from the operational taxonomic unit did not differ among groups. Note that ABX and ABX + T groups are absent as these groups did not have sufficient biodiversity for analyses, confirming the effectiveness of ABX in depleting gut microbiota. **(b)** Principal coordinate plot based on weighted UniFrac dissimilarity matrix of operational taxonomic units with each dot representing the microbiota of a sample and color-coded by treatment. **(c&d)** Stacked bar chart of microbial abundances at the phylum and genus levels, calculated as a percentage of total 16S rRNA reads within each group. **(e)** Bar-plot showing significant difference in the abundance of *Lactobacillus* genera. Data are *Mean* ± *SEM*; $*p < .05$, based on ANOVA with Tukey's post hoc test. *indicates a significant difference in MC and FC from female control mice. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

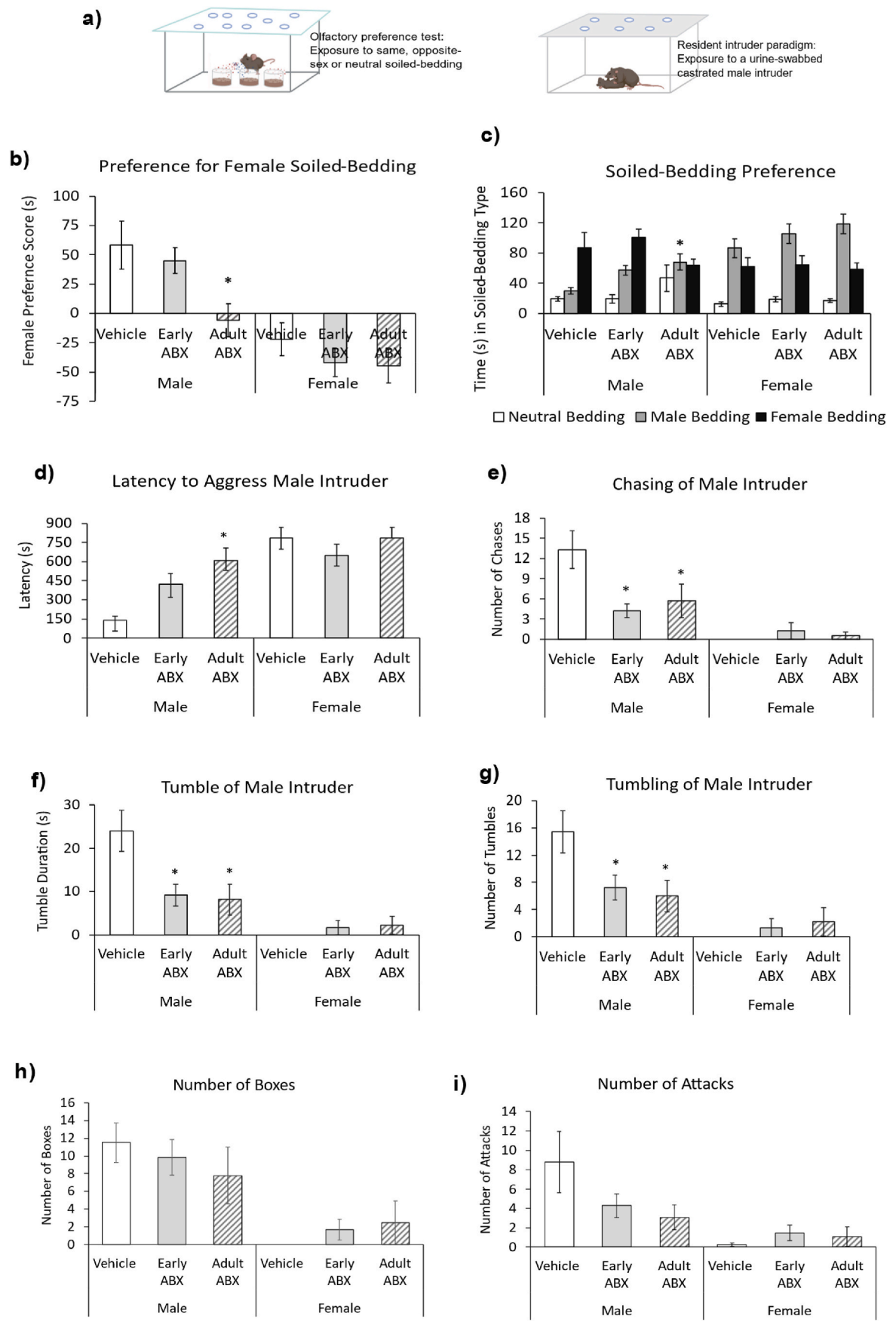

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Fig. 3. Antibiotic treatment decreases female odor preference and territorial aggression in male mice. (a) Behavioral testing took place between PND 85–90 and included the olfactory preference test, the resident-intruder paradigm and the sexual behavior test. Mice were tested following antibiotic (ABX) treatment in early development and adulthood. (**b, c**) On the olfactory preference test, treatment with ABX in adulthood led to a decreased female preference score (i.e., time spent in female-soiled bedding minus time in male-soiled bedding) in Adult ABX males due to an increased investigation of male-soiled bedding. (**d**) ABX treatment increased the latency to aggress in adult ABX males compared to vehicle-treated controls. Female groups did not significantly differ from each other. (**e-i**) Early ABX and Adult ABX males displayed reduced aggressive behaviors than vehicle control males. Females did not differ from one another regardless of ABX treatment status. Data are shown as mean ± SEM. *$p < .05$ indicates a significant difference when compared to vehicle controls.

number of face/body investigations (F (2, 68) = 3.56, $p = .034$, $\eta p^2 = 0.095$; Table 1), where Adult ABX mice investigated male intruders more than Early ABX ($p = .027$) but not vehicle control mice (all p values < .100). No sex by ABX treatment interaction was found on this measure ($p < .100$). Together, these results suggest the gut microbiota depletion via ABX, either during early life or in adulthood, does not affect adult sexual behaviors in mice.

3.3. ABX treatment in early development and adulthood decreased male territorial aggression in mice

To determine whether gut depletion via ABX affected aggression, mice were exposed to a castrated stimulus male swabbed with the urine of a sexually experienced male mouse in the resident-intruder paradigm. The expected sex difference was found for the latency to aggress and aggressive acts towards a urine-swabbed castrated male intruder, where male mice displayed more chasing, tumbling, and attacks than female mice (all p values < .001). Significant sex by ABX treatment interaction was found for the latency to aggress (F(2, 68) = 3.50, $p = .036$, $\eta p^2 = 0.093$; Fig. 3d). Specifically, Adult ABX male mice showed a longer latency to first aggress compared to vehicle control males ($p = .044$), while Early ABX males did not differ from vehicle control males on this measure (all p values > 0.100). We also observed significant sex by ABX treatment interaction for chasing frequency (F(2, 68) = 4.29, $p = .018$, $\eta p^2 = 0.112$), tumbling frequency (F(2, 68) = 3.57, $p = .034$, $\eta p^2 = 0.095$), and tumbling duration (F(2, 68) = 4.68, $p = .012$, $\eta p^2 = 0.123$). Specifically, ABX treatment in early development and adulthood decreased male aggression. Compared to vehicle controls, both Early and Adult ABX males showed reductions in these aggressive measures, while Adult but not Early ABX males took a longer time to display any aggressive behavior (i.e., latency to aggress; all p values < .05). Females did not differ from each other regardless of ABX condition (all p values > 0.100; Fig. 3d–g). As for the number of attacks or boxes toward a male intruder, no significant main effect of ABX nor an ABX by sex interaction was found (all p values > 0.100; Fig. 3h–i). Together, these results suggest a gut microbiota mediation of male-typical aggression and that ABX exposure during the early critical development period can have long-lasting effect on behavior.

Table 1
Investigations and sexual behaviors towards male and female stimulus mice following early development and adulthood antibiotic (ABX) treatment.

	Male			Female		
	Control ($n = 11$)	Early ABX ($n = 18$)	Adult ABX ($n = 14$)	Control ($n = 8$)	Early ABX ($n = 13$)	Adult ABX ($n = 11$)
Frequency of face/body investigation of male intruder *	24.40 ± 4.12	26.50 ± 3.04	35.78 ± 5.58	35.50 ± 6.24	25.92 ± 4.12	39.46 ± 4.54
Frequency of anogenital investigation of female intruder	24.50 ± 4.21	18.83 ± 2.58	23.07 ± 3.67	24.88 ± 5.88	21.54 ± 3.12	24.73 ± 4.38
Frequency of face/body investigation of female intruder	27.00 ± 3.43	25.22 ± 2.27	30.29 ± 3.92	36.75 ± 6.47	32.85 ± 4.03	40.46 ± 3.44
Frequency of mounting an estrus female	15.40 ± 3.62	8.83 ± 2.49	17.21 ± 3.17	2.13 ± 1.42	2.23 ± 1.03	3.36 ± 1.67
Frequency of thrusting an estrus female	59.50 ± 16.77	39.11 ± 13.41	62.00 ± 18.14	8.13 ± 5.42	9.00 ± 5.42	7.82 ± 5.35
Frequency of intromitting an estrus female	118.50 ± 42.63	58.00 ± 20.04	46.43 ± 25.93	19.25 ± 18.13	6.69 ± 5.20	4.36 ± 4.36

ABX, antibiotic; SEM, standard error of the mean; * indicates a main effect of ABX treatment and significant post hoc test when compared to Early ABX mice, but not vehicle-treated controls, $p < .05$; data are shown as mean ± SEM.

3.4. Cecal microbiota transplant but not testosterone rescues male-typical socio-sexual behaviors

As there is a significant role of androgens in the presentation of socio-sexual behaviors (Arnold, 2009) and there is a link between sex hormones and the gut microbiome (Ridlon et al., 2013), we assessed whether the decreased male-typical behaviors we observed in our previous experiments might be due to a reduction in circulating androgens and/or a lack of androgen-producing gut microbes (e.g., *Clostridium scindens*). We hypothesized that T capsule implants in mice receiving ABX treatment (ABX + T) would rescue ABX-induced behavioral deficit. Alternatively, restoring the gut composition via CMT with male control (MC) or female control(FC) cecal content would reverse ABX-induced socio-sexual behavioral deficits independent of androgens. Indeed, T implants increased androgen-sensitive seminal vesicle weight (F(4, 52) = 4.08, $p = .006$, $\eta p^2 = 0.239$), such that ABX + T males had heavier seminal vesicles than ABX, MC, and FC male mice (all p values < .05). In contrast, ABX + T males are larger, but not statistically so, from vehicle-treated control males ($p = .064$), suggesting that ABX + T male mice had similar-to-greater levels of circulating androgens comparable to vehicle male mice that received blank capsules. Furthermore, we observed significant increases in male-typical socio-sexual behaviors in a separate group of ABX-treated females with T capsule implants (see Supplementary Fig. A1), confirming the efficacy of the T treatment. It has also been demonstrated that this size T capsule (1.6-mm inner diameter, 3.2-mm outer diameter, 6-mm effective release length) is sufficient to restore androgen levels to pre-gonadectomy levels in castrated males (Zuloaga et al., 2008). However, when tested in the olfactory preference test, both ABX and ABX + T males showed a significantly lower female preference score ($p < .05$) compared to vehicle-treated controls. This is consistent with our finding in ABX-treated males from the previous experiment, indicating that T was insufficient to rescue this altered behavior. In contrast, MC and FC mice did not differ from vehicle-treated controls on this measure ($p = .547$ and .089, respectively; Fig. 4b). Interestingly, while there was a significant difference between the MC and ABX-only groups on sexual odor preference, the FC and ABX-only groups did not differ, suggesting that male,but not female, cecal transplant fully rescued ABX-induced decreased female preference score in male mice.

In the resident-intruder paradigm, significant effects of CMT and T treatments were found on the number of attacks (F(4, 52) = 3.49, $p = .013$, $\eta p^2 = 0.212$) and chasing duration ((F(4, 52) = 3.36, $p = .016$, $\eta p^2 = 0.205$) but not on the number of boxes (F(4, 52) = 1.49, $p = .219$). T

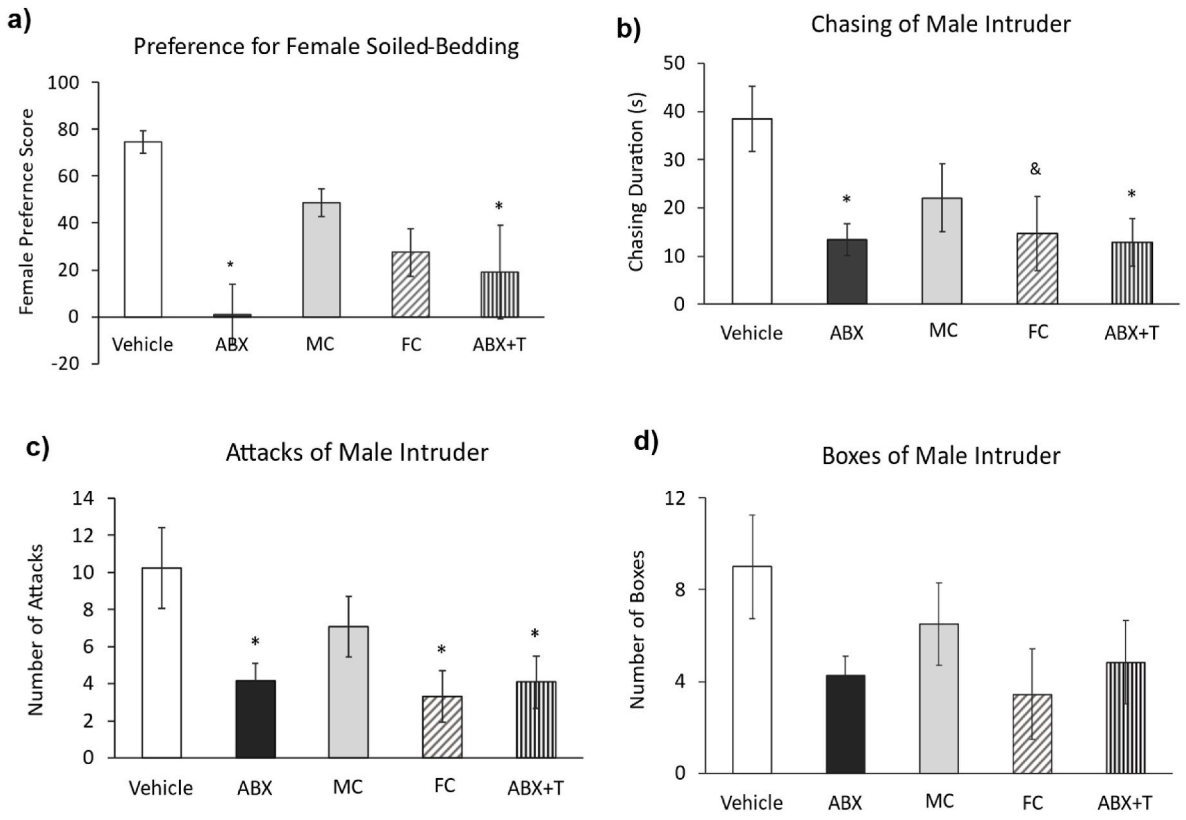

Fig. 4. Microbiota transplant, but not testosterone, prevents ABX-induced socio-sexual behavior deficits in ABX-treated male mice. Behavioral testing following cecal microbiota transplantation and T treatment occurred from PND 87–92. **(a)** ABX and ABX + T male mice displayed a decreased female preference score compared to vehicle-treated controls, consistent with behavioral results in Fig. 3. Male and female microbiota transplant rescued the ABX-induced olfactory preference in male recipient mice (i.e., MC and FC). ABX treatment, microbiota transplant, and T treatment interventions significantly affected the frequency and duration of aggressive bouts (i.e. chases and attacks; **c, d**) but not the number of boxes (**e**). ABX and ABX + T male mice showed decreased aggression compared to vehicle-treated control males. MC male mice did not differ from vehicle-treated controls. FC male mice displayed less number of attacks but were comparable to vehicle-treated controls males on other aggression measures. Data are shown as mean ± SEM; *$p < .05$, indicating a significant difference when compared to vehicle-treated controls. $^{\&}$ $p > .05$.

treatment in the absence of the microbiota was also insufficient to rescue male aggression. Specifically, ABX + T mice showed fewer attacks ($p = .025$) and shorter chases ($p = .040$) compared to vehicle-treated controls. Conversely, microbiota transplant reinstated aggression in previously ABX-treated males. Specifically, MC mice that received male microbiota were similar to vehicle-treated controls in the duration of chasing and the number of attacks (all p values $> .100$), whereas female cecal transplant partially rescued aggression in that FC mice showed near-equal levels of chasing ($p = .076$) but displayed lower number of attacks ($p = .035$) compared to vehicle-treated controls (Fig. 4c and d). Surprisingly, the MC and ABX-only groups did not statistically differ on these aggression measures (all p values $> .100$), suggesting that male cecal transplant has an intermediate phenotype. Boxing behavior did not differ among the groups (all p values $> .100$). Since male cecal transplant was more effective at restoring ABX-induced behavioral deficits, we assessed whether there are sex-specific bacteria in male control microbiota that promoted male-typical behaviors by correlating the major bacterial phyla with sexual odor preference and aggressive behaviors. There were no significant associations between aggression and the three major phyla (Firmicutes, Bacteriodetes, and Proteobacteria) (all p values > 0.100; Table 2), suggesting that sex-specific gut microbes may not influence these behaviors. Together, these results suggest that

Table 2
Spearman correlation between socio-sexual behaviors and three major bacteria phyla.

		Attacks	Chasing Duration	Female Preference Score	Firmicutes	Bacteroidetes
Attacks	Spearman's rho	–				
	p-value	–				
Chasing Duration	Spearman's rho	.841	–			
	p-value	<.001	–			
Female Preference Score	Spearman's rho	.365	.363	–		
	p-value	.005	.006	–		
Firmicutes	Spearman's rho	.156	.122	.194	–	
	p-value	.489	.588	.386	–	
Bacteroidetes	Spearman's rho	.225	.168	.204	.842	–
	p-value	.315	.455	.361	<.001	–
Proteobacteria	Spearman's rho	−.165	−.020	.157	.640	.594
	p-value	.464	.930	.485	.001	.004

Spearman correlation revealed no association between major phyla and socio-sexual behaviors, $p > .100$.

the commensal microbiota is necessary for the display of male-typical aggression and that the gut microbiome's influence on socio-sexual behaviors is independent of sex hormones.

4. Discussion

The present study evaluated the effects of gut microbiota depletion in early development versus adulthood on socio-sexual behaviors as well as whether the gut microbiome acts via androgens to influence these behaviors. Our results show that ABX treatment induced alterations in the microbiota composition, and these alterations returned to baseline when treated with CMT. Our findings also suggest that the gut microbiome mediates male-typical aggression and olfactory preference but not copulatory behaviors in male mice. ABX treatment did not influence socio-sexual behaviors in female mice. Furthermore, microbiota transplant but not T treatment rescued the ABX-induced behavioral deficits in adult male mice. Together, these results suggest a sex-dependent gut microbiome mediation of socio-sexual behaviors that is independent of androgens.

ABX-mediated depletion of the microbiota in male mice induced significant gut dysbiosis as well as behavioral changes in mice, which are consistent with previous studies using similar ABX regimens in rodents (Desbonnet et al., 2015; Reikvam et al., 2011; Sylvia et al., 2017) and supports the idea that a non-pathogenic bacterial population is critical for brain development and behavior. In the current study, ABX treatment during adulthood decreased female preference score in male mice, as ABX-treated male mice spent more time investigating male-soiled bedding instead of female-soiled bedding. Olfactory behaviors such as sexual odor preference play a critical role in mate choice and reproductive success (Huo et al., 2018), but the impact of gut microbiota on olfactory behaviors has not been previously assessed in mice. However, in *Drosophila*, ABX treatment has been shown to reduce olfactory responses that were partly restored by probiotic treatment (Slankster et al., 2019). In the present study, male-typical sexual behaviors (e.g., mounting and thrusting) towards an estrus female remained altered by ABX treatment in male mice. This suggests that the observed decrease in olfactory preference in male mice is not indicative of a decrease in sexual interest but rather related to odor or pheromone processing. For example, a thinner olfactory cilia layer and decreased cellular level transduction have been reported in germ-free mice compared to conventional mice with healthy microbiota (François et al., 2016). Given the novelty of this finding, future research may look into the relationship between the gut microbiome and the olfactory epithelium/vomeronasal organ, as well as the effects of this connection on behavior, in order to better understand the role of microbiota in behavior.

Our results also indicate that microbiota depletion decreased territorial aggression in ABX-treated male mice when exposed to a castrated male intruder, consistent with a previous study that demonstrated a sex-dependent effect of microbiota disruption on aggression among Siberian hamsters (Sylvia et al., 2017). In that study, two 7-day ABX treatments followed by a recovery phase reduced male aggression (Sylvia et al., 2017). In the present study, a continuous 2–3 weeks of adulthood ABX treatment caused a marked decrease in territorial aggression among male mice. Furthermore, male mice that received ABX in early development (i.e., embryonic day 16-PND 21) via maternal exposure also displayed decreased male aggression in adulthood, although the early ABX mice had sufficient time (64+ days) to recover from ABX exposure. This suggests that early-life ABX exposure may have a prolonged change in the gut microbiota composition and behavior. Indeed, previous studies have shown that maternal ABX treatment during pregnancy alters offspring's microbiota composition and immunity, resulting in behavioral deficits in adulthood (Nyangahu et al., 2018; O'Connor et al., 2021). Together, the reductions in socio-sexual behaviors are consistent with previous lines of research linking microbiota depletion to social deficits such as social avoidance, increased anxiety and decreased social activity in mice (Buffington et al., 2016; Desbonnet et al., 2014; Wu et al., 2021).

In mice, androgen action within sexually dimorphic neural pathways (e.g., the accessory olfactory system) organizes the brain to promote male-typical behaviors such as female odor preference and territorial aggression. For example, gonadectomy or ablation of the vomeronasal organ (an androgen-sensitive organ responsible for pheromone processing) has been associated with decreased preference for opposite-sex odors and male aggression (Bodo and Rissman, 2008; Cross et al., 2021; Schellino et al., 2016). It is possible that these neural pathways may be sensitive to microbiota perturbations and/or that gut microbiota depletion may alter the hypothalamic-pituitary-gonadal (HPG) axis, which regulates the production and secretion of sex hormones, including androgens (Organski et al., 2021). For instance, a link between the microbiota and gonadal hormones has been reported—namely, gut microbe *Clostridium scindens* can produce androgens from glucocorticoids (Ridlon et al., 2013); thus, depletion of the microbiota may alter androgen-producing microbes and the HPG axis (Tetel et al., 2018), leading to alterations in circulating androgens and subsequently on male aggression and olfactory preference. We anticipated that T treatment in male mice receiving ABX treatment would compensate for these ABX-microbial effects on behaviors. However, T treatment was insufficient to reverse the ABX-induced socio-sexual behavior deficits in male mice, even though an increased seminal vesicle weight (which correlates with increased circulating testosterone) was observed. One explanation for this observation is that ABX treatment likely masked the effect of T on behavior (Popoola et al., 2014), rendering T inadequate to restore male aggression. For example, ABX and/or the absence of the gut microbiota may inhibit the abundance of circulating androgens and/or receptors, and merely increasing circulating androgens may not be sufficient to overcome these effects of ABX (Markle et al., 2013). Even so, this possible masking effect of ABX seems unlikely in males, given the responsiveness of the seminal vesicles to T treatment. Moreover, T treatment produced more male-typical behaviors in our ABX-treated females as expected (Martel and Baum, 2009; see Supplementary Fig. A1), thus confirming the effectiveness of the androgen treatment, although circulating T levels were not measured in the current study. However, it remains possible that ABX treatment could mask the effects of T. For example, the gonadotropin-releasing hormone (GnRH) component of the HPG axis has been associated with gut microbiota alterations through pro-inflammatory pathways induced by lipopolysaccharides (LPS) (Im et al., 2012; Lee et al., 2019; Organski et al., 2021). Hence, a possible connection between microbiota depletion and highly permeable gut metabolites (e.g., LPS, butyrate) might interact with the neuroendocrine HPG pathway to affect circulating T, rendering it incapable of rescuing ABX-induced behavioral deficits in ABX-treating male mice.

Alternatively, gut microbiota may mediate socio-sexual behaviors independently of sex hormones. In the current study, gut bacteria transplantation with male or female control (healthy) cecal content rescued ABX-induced olfactory preference, suggesting CMT may have maintained bacterial balance and function in odor processing. However, only male microbiota—not female microbiota transplantation—effectively rescued territorial aggression in ABX-treated male mice. Indeed, restructuring a depleted microbiota via cecal or fecal microbiota transplantation has previously been shown to reverse gut dysbiosis (Hamilton et al., 2013) and associated behavioral deficits (Schmidt et al., 2020; Tillmann et al., 2019; Wu et al., 2021) and thus aligns with the current finding. CMT restored bacterial balance in ABX-treated male mice and so may have potentially acted through immune (e.g., gut-mediated cytokines) and neuroendocrine pathways (e.g., via the vagus nerve or hypothalamic-pituitary-adrenal axis (HPA)] to rescue ABX-mediated behavioral deficits. For instance, gut dysbiosis is highly associated with inflammation and social behavioral deficits (Agranyoni et al., 2021; Elias-Oliveira et al., 2020). It has previously been shown that ABX treatment increases intestinal barrier permeability (Desbonnet et al., 2015; Feng et al., 2019), allowing microbial

metabolites (e.g., short-chain fatty acid) to enter the circulation, leading to increased systemic inflammation (Gwak and Chang, 2021; Martin et al., 2018). Notably, inflammation plays an important role in shaping social processes and contributing to social impairments (Agranyoni et al., 2021; Moieni and Eisenberger, 2018) as well as olfactory dysfunction in some cases (Rouyar et al., 2019; Torabi et al., 2020). Thus, it is possible that CMT improved the gut integrity of the intestinal barrier (Segal et al., 2020), subsequently rescuing sex-typical behaviors in male mice.

The stress response HPA axis may be another mechanism driving the recovery of socio-sexual behaviors in male mice via CMT. For instance, Wu et al. (2021) reported elevated corticosterone levels in ABX-treated and germ-free mice, which was associated with decreased social activity, suggesting that microbial perturbation via ABX potentially activated the HPA axis in mice in the present study. Furthermore, gut metabolites may activate the vagus nerve to communicate directly with the brain to affect behavior; however, Wu et al. (2021) noted that the vagus nerve is not involved in the social impairment resulting from microbiota depletion after performing vagotomy in ABX mice (Wu et al., 2021). Nevertheless, it is still likely that the microbiome communicates with the brain via the gut-brain axis to mediate behavior. We hypothesize that multiple systems may be involved in the CMT mediation of male-typical socio-sexual behaviors, likely through gut-immune or neuro-modulatory pathways, for which future research is necessary to gain mechanistic insights. Moreover, antibiotics that target the gut microbiota may affect the microbiota in other areas of the body (e.g., mouth), and the systemic effects of absorbed antibiotics might affect behavior independently of their actions on microorganisms.

The full rescue of sexual odor preference and the intermediate rescue of aggression in ABX-treated male mice suggested that male microbiota may contain a high abundance of specific microbes associated with male-typical behaviors as found in dogs (i.e., *Lactobacillus* and *Dorea*; Kirchoff et al., 2019), which may not be present in the female microbiota. However, we did not find significant correlations between bacteria and these socio-sexual behaviors. The phyla Tenericutes and Cyanobacteria have previously been positively associated with attacking behavior where ABX treatment decreased aggression in male and female hamsters (Sylvia et al., 2017). In the current study, a small percentage (less than 1%) of mice's microbiota was made up of Cyanobacteria, but none of Tenericutes. Specifically, the microbiota composition of mice in the present study was dominated by the phyla Firmicutes, Bateriodetes, Proteobacteria and Actinobacteria and no sex by treatment effect was observed in both untreated control males and females and that of ABX-treated male mice who received CMT; hence it is unlikely that ABX treatment affected the abundance of Cyanobacteria. Since there is evidence of species and sex differences in aggression behavior (Bodo and Rissman, 2008; Scotti et al., 2008) and the microbiota composition (Org et al., 2016), Cyanobacteria may not be associated with aggression in mice. Hence, sex-specific gut bacteria may not influence aggression in mice as they do in other species.

Although it is unclear how female microbiota partially rescued behavior in male mice, sex-specific differences arising from the presence of X or Y chromosomes and/or sex hormones may explain this finding. For instance, male mice evolve with a male-typical microbiota; hence, female microbiota transplant may not function fully normally in ABX-treated males, pointing to host-specificity effects. Yurkovetskiy et al. (2013) showed that irrespective of the sex of the donor, the gut microbiota distinctly segregates according to the sex of the recipient/host. In other words, the intestines of male and female mice may accommodate microbiota transplantation differently (Wang et al., 2016). Considering that territorial aggression, for instance, is more typical in male mice, female microbiota in male recipients may require an extended period to restructure to rescue male-typical behaviors. Sex differences in the immune system may also impact microbiota transplantation. For example, males have a higher percentage of regulatory T cells (Treg) irrespective of whether they received microbiota transplants from male or female donors (Fransen et al., 2017). Thus, transplanting either male or female microbiota to sex-matched or opposite-sex mice may produce different behavioral changes. Therefore, it is important for future studies assessing microbiota transplantation as a therapeutic intervention to consider the complex interaction between donor and recipient sex on the microbiota.

Taken together, the present study suggests that sex-dependent gut microbes contribute to socio-sexual behaviors in mice and that the effect of the gut microbiome on these behaviors is independent of androgens.

4.1. Limitations

While the present study explored the impact of the gut microbiota depletion on socio-sexual behaviors in mice, we cannot ascertain the extent to which the gut microbiome mediates female-typical socio-sexual behaviors (e.g., maternal care and aggression). In the resident intruder and sexual behavior paradigm, the estrus cycle of experimental female mice was not examined, and these mice were not exposed to sexually experienced males. Thus, alterations in female socio-sexual behaviors may emerge when in behavioral estrus and presented with a reproductive male. Similarly, female aggressive behavior was not assessed in paradigms where females display heightened aggression (e.g., during postpartum). Thus, future studies using paradigms such as maternal aggression tests are needed to confirm whether the microbiome mediates aggression in female mice. Lastly, different mechanisms may be driving differences in behavioral effects likely through gut-immune and neuro-modulatory pathways in which T may or may not be involved. For instance, the variation in antibiotic duration in the current study, as well as non-specific influence ofantibiotics may contribute to behavioral deficits. Follow-up microbiota manipulation studies during early critical phases of brain development are likely to generate mechanistic insights into gut microbiota mediation of behavior.

4.2. Conclusion

The present study has elucidated for the first time the role of the gut microbiome on socio-sexual behavior in mice. Depletion of the microbiota via ABX in early development decreased territorial aggression, while treatment in adulthood decreased sexual odor preference and aggression in male but not female mice. Conversely, sexual behaviors remained unaltered in both male and female ABX-treated mice. Furthermore, CMT but not T treatment was sufficient to prevent ABX-induced gut dysbiosis and socio-sexual behavioral deficits in ABX-treated male mice. Our findings strengthen the initial report on the relationship between the microbiota and aggression in ABX-treated rodents, and suggest a healthy microbiome is necessary for the full expression of sex-typical socio-behavior. The study further advances knowledge on the short- and long-term impacts of ABX treatment on sex-typical behaviors, which has overarching implications for how the gut microbiome may influence the brain and behavior in a sex-dependent manner.

Author contributions

SS and AS-G conceived the project, designed both experiments and analyzed data. SS, YM, FFB, LAM, and LJ assisted with behavior assays, including data collection. The manuscript was written by SS, and review/editing was done by SS, AS-G, FRB, KH and YM. AS-G and FRB secured funding for the project.

Declaration of competing interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2023.100637.

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